

Sildenafil (Viagra) Induces Neurogenesis and Promotes Functional Recovery After Stroke in Rats

Ruilan Zhang, MD; Ying Wang, MD; Li Zhang, MD; Zhenggang Zhang, MD, PhD; Wayne Tsang, BS; Mei Lu, PhD; Lijie Zhang, MD; Michael Chopp, PhD

Background and Purpose—We tested the hypothesis that sildenafil, a phosphodiesterase type 5 (PDE5) inhibitor, promotes functional recovery and neurogenesis after stroke.

Methods—Male Wistar rats were subjected to embolic middle cerebral artery occlusion. Sildenafil (Viagra) was administered orally for 7 consecutive days starting 2 or 24 hours after stroke onset at doses of 2 or 5 mg/kg per day. Ischemic rats administered the same volume of tap water were used as a control group. Functional outcome tests (foot-fault, adhesive removal) were performed. Rats were killed 28 days after stroke for analysis of infarct volume and newly generated cells within the subventricular zone and the dentate gyrus. Brain cGMP levels, expression of PDE5, and localized cerebral blood flow were measured in additional rats.

Results—Treatment with sildenafil significantly ($P < 0.05$) enhanced neurological recovery in all tests performed. There was no significant difference of infarct volume among the experimental groups. Treatment with sildenafil significantly ($P < 0.05$) increased numbers of bromodeoxyuridine-immunoreactive cells in the subventricular zone and the dentate gyrus and numbers of immature neurons, as indicated by β -tubulin (TuJ1) immunoreactivity in the ipsilateral subventricular zone and striatum. The cortical levels of cGMP significantly increased after administration of sildenafil, and PDE5 mRNA was present in both nonischemic and ischemic brain.

Conclusions—Sildenafil increases brain levels of cGMP, evokes neurogenesis, and reduces neurological deficits when given to rats 2 or 24 hours after stroke. These data suggest that this drug that is presently in the clinic for sexual dysfunction may have a role in promoting recovery from stroke. (*Stroke*. 2002;33:2675-2680.)

Key Words: neurogenesis ■ recovery of function ■ sildenafil ■ stroke, cardioembolic ■ rats

Nitric oxide (NO) is a potent activator of soluble guanylate cyclase and causes cGMP formation in target cells.¹ Phosphodiesterase type 5 (PDE5) enzyme is highly specific for hydrolysis of cGMP and is involved in regulation of cGMP signaling.^{2,3} Sildenafil is a novel inhibitor of PDE5 and causes intracellular accumulation of cGMP.³

Administration of an NO donor to rats with stroke significantly increases brain levels of cGMP, induces cell genesis, and improves functional recovery.⁴ Functional recovery may be partly due to increases in levels of cGMP resulting from administration of an NO donor. Therefore, we hypothesized that administration of sildenafil, a PDE5 inhibitor, to rats subjected to stroke enhances improvement of neurological outcome during stroke recovery. In the present study we tested this hypothesis in a rat model of focal cerebral embolic ischemia.

Materials and Methods

Sildenafil is a weak basic compound, which is therefore only partially ionized at physiological pH and has a half-life of 0.4 hour

in rats.⁵ A film tablet of Viagra (contain 100 mg sildenafil, purchased commercially) was weighed and powdered.

Animal Model

Male Wistar rats weighing 320 to 380 g were used in the present study. The middle cerebral artery (MCA) was occluded by placement of an embolus at the origin of the MCA.⁶

Experimental Protocols

In protocol 1, to examine whether administration of sildenafil affects cell proliferation and neurological behavior, sildenafil at 2 mg/kg ($n=10$) or 5 mg/kg ($n=9$) dissolved in 3 mL of tap water was administered orally to rats 2 hours after MCA occlusion and daily for an additional 6 days. Another group of the ischemic rats ($n=10$) was treated orally with sildenafil (2 mg/kg) 24 hours after MCA occlusion and daily for an additional 6 days. Ischemic rats ($n=9$) were treated with the same volume of tap water as a control group. Functional tests were performed and body weight was measured before ischemia and at 4, 7, 14, 21, and 28 days after onset of MCA occlusion. All rats were killed 28 days after MCA occlusion. In protocol 2, to examine whether administration of sildenafil affects brain cGMP levels, nonischemic rats were treated with sildenafil at 2 mg/kg ($n=6$) or tap water ($n=10$) for 7 days. These rats were killed

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From the Department of Neurology, Henry Ford Health Sciences Center, Detroit, Mich (R.Z., Y.W., L.Z., Z.Z., W.T., M.L., L.Z., M.C.), and Department of Physics, Oakland University, Rochester, Mich (M.C.).

Correspondence to Michael Chopp, PhD, Henry Ford Hospital, Department of Neurology, 2799 W Grand Blvd, Detroit, MI 48202. E-mail: chopp@neuro.hfh.edu

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1 hour after the last treatment for measurements of brain levels of cGMP. In protocol 3, to examine the effects of sildenafil on cerebral blood flow (CBF) and blood pressure, nonischemic rats ($n=6$) were treated orally with sildenafil, and local CBF and mean arterial blood pressure were measured starting at 30 minutes and continuing for 180 minutes after administration of sildenafil. In protocol 4, to examine brain PDE5, nonischemic rats and ischemic rats were killed at 2, 4, 24, 48, and 168 hours after the onset of ischemia ($n=3$ for each time point). Reverse transcription (RT)-polymerase chain reaction (PCR) was performed to detect PDE5 in brain tissue.

cGMP Measurement in Brain Tissue

Levels of cGMP were measured with the use of a commercially available low-pH immunoassay kit (R&D Systems Inc). The sensitivity of the assay was approximately 0.6 pmol/mL for the nonacetylated procedure. The brain was rapidly removed, and the cortex and the cerebellum were separated.⁴ The brain tissue was weighed and homogenized in 10 volumes of 0.1N HCl containing 1 mmol/L 3-isobutyl-1-methylxanthine.

RT-PCR Analysis

To examine the presence of PDE5 in rat brain tissue, we synthesized primers for PDE5A1 and PDE5A2 according to published sequences.⁵ The 5' primer 5'-AAACTCGAGCAGAAACCCGCCGCA-ACACC-3' and the 3' primer 3'-GCATGAGGACTTTGAG-CGACAGAGC-3' amplified a cDNA fragment coding for N-terminal regions of rat PDE5A1.⁵ The 5' primer 5'-ACCTCTGCTATGTTGCCCTTTGC-3' and the 3' primer 3'-GCATGAGGACTTTGAGCGACAGAGC-3' amplified a cDNA fragment coding for rat PDE5A2.⁵

For cDNA synthesis, total RNA extracted from brain tissue was reverse transcribed.⁷ The samples were denatured at 95°C for 2 minutes and then amplified for 40 cycles. Each cycle consisted of denaturation at 95°C for 30 seconds, annealing at 62°C for 1 minute, and extension at 72°C for 2 minutes. The samples (30 μ L per well) were electrophoresed on 1.5% agarose containing ethidium bromide.

Body Weight Loss

Animals were weighed before and at 4, 7, 14, 21, and 28 days after embolic ischemia. Body weight loss is presented as a percentage of preischemic body weight.

Foot-Fault Test

Rats were tested for placement dysfunction of forelimbs with the modified foot-fault test⁸ before ischemia and at 4, 7, 14, 21, and 28 days after embolic ischemia. Rats were set on an elevated hexagonal grid of different sizes and placed their paws on the wire while moving along the grid. With each weight-bearing step, the paw may fall or slip between the wire. The total number of steps (movement of each forelimb) that the rat used to cross the grid was counted, and the total number of foot-faults for each forelimb was recorded.

Adhesive Removal Test

An adhesive removal test was used to measure somatosensory deficits⁹ and was performed before MCA occlusion and at 4, 7, 14, 21 and 28 days after MCA occlusion.

Bromodeoxyuridine Labeling

Bromodeoxyuridine (BrdU) was used to measure cell proliferation.¹⁰ Animals received daily intraperitoneal injections of BrdU (50 mg/kg; Sigma) on the day of stroke and subsequently for 14 consecutive days. Cell proliferation in the subventricular zone and dentate gyrus was measured in rats killed at 28 days (in experimental protocol 1, all 4 groups) after ischemia.

Immunohistochemistry

For BrdU immunostaining, DNA was first denatured by incubating brain sections (6 μ m) in 50% formamide 2 \times SSC at 65°C for 2 hours and then in 2N HCl at 37°C for 30 minutes.^{4,11} Sections were then

fixed with Tris buffer and treated with 1% of H_2O_2 to block endogenous peroxidase. Sections were incubated with a primary antibody to BrdU (1:100) at room temperature for 1 hour and then incubated with biotinylated secondary antibody (1:200, Vector) for 1 hour. Reaction product was detected with the use of 3'3'-diaminobenzidine-tetrahydrochloride (DAB; Sigma). For β III-tubulin (TuJ1) immunostaining, which identifies immature neurons,¹² coronal sections were incubated with the antibody against TuJ1 (1:1000) at 4°C overnight and were then incubated with a biotinylated horse anti-mouse immunoglobulin antibody at room temperature for 30 minutes. Double immunofluorescent staining for BrdU and TuJ1 was performed to determine whether BrdU-immunoreactive cells express neuronal phenotype on the coronal sections.

Image Analysis and Quantification

Measurements of BrdU-immunoreactive cells were performed on paraffin-embedded 6- μ m-thick sections.¹¹ BrdU-immunostained sections were digitized with the use of a $\times 40$ objective (Olympus BX40) via the MCID computer imaging analysis system (Imaging Research). BrdU-immunoreactive nuclei were counted on a computer monitor to improve visualization and in 1 focal plane to avoid oversampling.

All BrdU-immunoreactive-positive nuclei were counted in both the ipsilateral and contralateral walls of the lateral ventricle of the subventricular zone and in the dentate gyrus.¹¹ For the subventricular zone, every 40th coronal section was selected from each rat for a total of 7 sections between anteroposterior +10.6 mm of the genu corpus callosum and anteroposterior +8.74 mm of the anterior commissure crossing. For the dentate gyrus, every 30th coronal section was selected from each rat for a total of 8 sections between anteroposterior +5.86 mm and anteroposterior +2.96 mm of the granule cell layer. BrdU-immunoreactive nuclei in the subventricular zone and in the dentate gyrus are presented as the number of the cells per square millimeter (mean \pm SE). Density values for the 7 sections (subventricular zone) and 8 sections (dentate gyrus) were averaged to obtain a mean density value for each animal.⁴

Numbers of TuJ1-immunoreactive cells were counted in the subventricular zone and striatum, and data are presented as the number of TuJ1-immunoreactive cells per section (mean \pm SE).

Monitoring of Relative Erythrocyte Flow Velocity

Relative erythrocyte flow velocity was measured by laser-Doppler flowmetry (PeriFlux PF4 flowmeter; Perimed AB) in the tissue under the laser-Doppler flowmetry probe.¹³ A burr hole 1.5 mm in diameter was drawn on the skull 2 mm posterior to the bregma and 6 mm lateral to midline.¹³ The dura was left intact. After the application of mineral oil onto the burr hole, the probe was placed 0.5 mm above the dural surface. Relative flow velocities were measured 30 minutes after administration of sildenafil. This measurement reflects relatively localized CBF.¹⁴ Values of flow velocities are presented as a percentage of the contralateral hemispheric values.

Measurements of Infarct Volume

Measurement of infarct volume was measured on 7 hematoxylin and eosin-stained coronal sections with the use of a Global Laboratory Image analysis program (Data Translation).¹⁵ Briefly, the area of both hemispheres and the infarct area (mm²) were calculated by tracing the area on the computer screen. Infarct volume (mm³) was determined by multiplying the appropriate area by the section interval thickness.¹⁶ The infarct volume is presented as the percentage of infarct volume of the contralateral hemisphere (indirect volume calculation).¹⁶

Statistical Analysis

For analysis of neurological functional recovery and body weight, the generalized estimation equations (GEE) analysis¹⁷ approach was used instead of ANOVA because the data did not meet assumptions of normality and equal variance for ANOVA. A paired t test or signed rank test was used to test the difference in cell proliferation

TABLE 1. Density of Newborn Cells in Brain

Groups	Subventricular Zone		Dentate Gyrus	
	Ipsilateral	Contralateral	Ipsilateral	Contralateral
Sildenafil 2 mg/kg, 2 h	383±23.44*	296±18.74	55±3.89*	55±2.10†
Sildenafil 5 mg/kg, 2 h	437±32.97†	312±23.79*	59±5.28*	58±5.38*
Sildenafil 2 mg/kg, 24 h	374±18.07*	289±24.54	57±4.21*	56±4.76*
Control	285±32.68	246±18.54	44±2.86	42±3.01

Density of newborn cells is presented as mean±SEM number of BrdU-immunoreactive cells per mm².

* $P<0.05$, † $P<0.01$ vs control group.

between ipsilateral and contralateral regions of subventricular zone, dentate gyrus, and striatum. The GEE analysis approach was used to study the treatment effect on cell proliferation in the ipsilateral and contralateral subventricular zone regions, dentate gyrus, and striatum. All values are presented as mean±SE. Statistical significance was set at $P<0.05$.

Results

Effects of Sildenafil on Cell Proliferation

Ischemic rats treated with sildenafil (2 or 5 mg/kg) initiated at 2 or 24 hours after stroke had significant ($P<0.05$) increases

in numbers of BrdU-immunoreactive cells in the dentate gyrus of both hemispheres (Table 1) compared with control rats. Treatment with sildenafil at a dose of 2 mg/kg (at 2 or 24 hours) significantly ($P<0.05$) increased numbers of BrdU-immunoreactive cells in the ipsilateral subventricular zone (Table 1), and the 5 mg/kg dose (at 2 hours) significantly ($P<0.05$) increased numbers of BrdU-immunoreactive cells in the subventricular zone of both hemispheres (Table 1) compared with numbers of BrdU-immunoreactive cells in control rats.

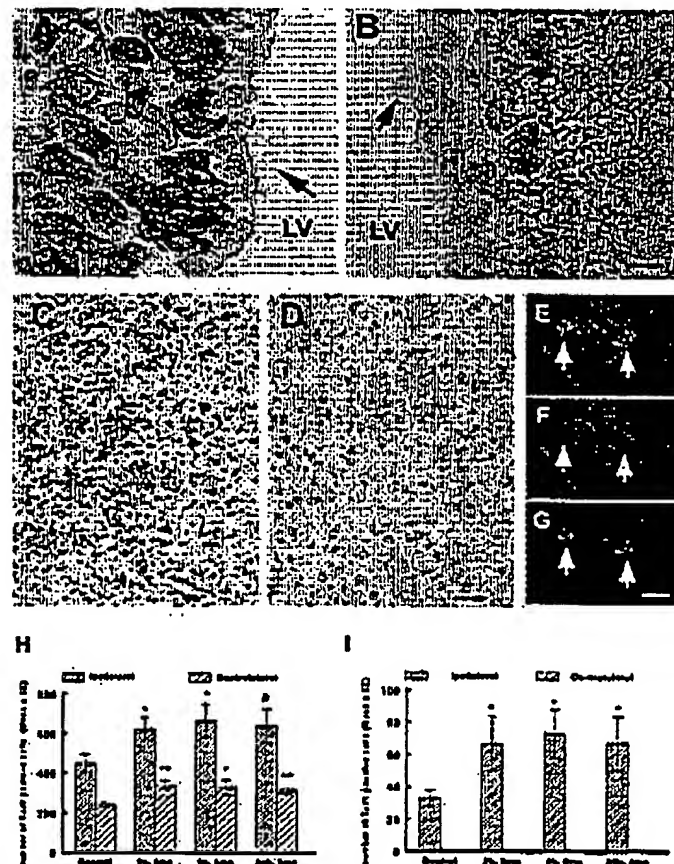


Figure 1. Treatment with sildenafil increased TuJ1-immunoreactive cells 28 days after ischemia. A, From a representative rat, robust increases in numbers of TuJ1-immunoreactive cells in the ipsilateral subventricular zone compared with the contralateral subventricular zone (B) are shown. Ependymal cells (arrows in A and B) were not TuJ1 immunoreactive. TuJ1-immunoreactive cells exhibited cluster in the ipsilateral striatum (C) compared with the homologous tissue in the contralateral hemisphere (D). Double immunostaining with antibodies against TuJ1 and BrdU shows that BrdU-immunoreactive cells (E and G, green, arrows) were TuJ1 immunoreactive (E and F, red, arrows). E is a merged image from F and G. H and I show quantitative data of numbers of TuJ1-immunoreactive cells in the subventricular zone ($n=6$ in each group) and striatum ($n=6$ in each group), respectively. * $P<0.05$, ** $P<0.01$, # $P=0.05$ vs control group. LV indicates lateral ventricle. Bars=10 µm in B and G and 20 µm in C.

TABLE 2. Foot-Fault Test

Groups	% of Foot-Faults					
	Before ischemia	4 d	7 d	14 d	21 d	28 d
Sildenafil 2 mg/kg, 2 h	1.1±0.01	22.81±3.1	15.2±1.6†	13.2±1.2†	9.1±1.5†	8.2±1.4
Sildenafil 5 mg/kg, 2 h	1.02±0.02	17.8±2.9	16.8±1.4*	14.8±2.1*	8.5±1.0†	7.0±1.4
Sildenafil 2 mg/kg, 24 h	1.03±0.03	25.3±3.8	14.4±1.0†	10.0±0.5†	8.0±0.5†	5.9±0.8*
Control	1.08±0.07	31.4±3.4	24.8±3.0	22.0±2.8	18.4±2.7	11.8±1.0

Values are mean±SE for specified number of days after ischemia.

* $P<0.05$, † $P<0.01$ vs control group.

Effects of Sildenafil on Immature Neurons

Administration of sildenafil robustly increased number of TuJ1-immunoreactive cells in the ipsilateral subventricular zone (Figure 1A) and striatum (Figure 1C). TuJ1-immunoreactive cells exhibited clusters in the ipsilateral striatum (Figure 1C). Some of the TuJ1-immunoreactive cells were BrdU immunoreactive (Figure 1E to 1G). Quantitative measurements revealed that administration of sildenafil at a dose of 2 or 5 mg/kg significantly ($P<0.05$) increased numbers of TuJ1-immunoreactive cells in the ipsilateral and contralateral subventricular zones compared with the number in control rats (Figure 1H). Treatment with sildenafil also significantly increased the number of TuJ1 cells in the ipsilateral striatum compared with homologous tissue in the contralateral hemisphere and in the ipsilateral striatum of control rats (Figure 1I).

Effects of Sildenafil on Neurological Outcome

The ischemic rats treated with sildenafil at a dose of 2 or 5 mg/kg significantly improved performance on the foot-fault test (Table 2) and the adhesive removal test (Table 3) during 4 to 21 days compared with control rats when treatment was initiated at 2 hours after onset of ischemia. In addition, treatment with sildenafil at doses of 2 and 5 mg/kg significantly reduced animal body weight loss (Table 4). In contrast, infarct volumes measured 28 days after ischemia were not significantly different among these groups (Table 5), suggesting that infarct volume does not contribute to improvement of functional recovery. We also administered sildenafil at a dose of 2 mg/kg to the ischemic rats starting at 24 hours after onset of ischemia. Ischemic rats receiving sildenafil exhibited significant ($P<0.05$) improvements on the foot-fault (Table 2) and adhesive removal (Table 3) tests during 7 to 28 days after stroke. Rats treated with sildenafil also showed a significant ($P<0.05$) reduction in body weight loss at 4, 7, 14, 21, and 28 days after ischemia (Table 4). However, there were no significant differences in infarct volume between

ischemic animals treated with sildenafil and animals in the control group (Table 5).

Effects of Sildenafil on cGMP

The cerebellar levels of cGMP (Figure 2A, control) were higher than the cortical (Figure 2B, control) levels in nonischemic control rats, which is consistent with previous studies.⁴ Treatment with sildenafil at a dose of 2 or 5 mg/kg for 7 days significantly ($P<0.05$) increased the cortical levels of cGMP (Figure 2B) compared with levels in the control group.

Effects of Sildenafil on Localized CBF

Administration of sildenafil at a dose of 2 mg/kg to nonischemic rats significantly increased localized CBF levels compared with the control rats (Figure 3). Significantly increased localized CBF persisted for 70 minutes after administration of sildenafil (Figure 3).

PDES in Rat Brain

RT-PCR analysis revealed both PDE5A1 (257 bp) and PDE5A2 (149 bp) transcripts in nonischemic rat brain tissue, indicating the presence of PDES (data not shown). Levels of PDE5A1 and PDE5A2 mRNA measured by band density ($n=3$ for each time point) did not show a statistical difference after MCA occlusion compared with the nonischemic rats.

Discussion

The present study demonstrates that treatment of focal cerebral ischemia in rats with sildenafil significantly improved recovery of neurological outcome and significantly increased numbers of BrdU- and TuJ1-immunoreactive cells in ischemic brain. In addition, administration of sildenafil significantly increased cortical levels of cGMP. Therefore, our data suggest that increased cGMP levels resulting from administration of sildenafil may mediate enhanced neurological outcome.

TABLE 3. Adhesive Removal Test

Groups	Seconds					
	Before ischemia	4 d	7 d	14 d	21 d	28 d
Sildenafil 2 mg/kg, 2 h	7.0±0.1	85.4±9.8	41.9±8.4†	27.8±3.9†	23.6±5.1*	15.9±3.8
Sildenafil 5 mg/kg, 2 h	16.7±0.4	100.7±9.2	70.7±10.8*	38.8±7.9†	26.0±6.6†	14.8±3.9
Sildenafil 2 mg/kg, 24 h	8.8±0.3	102±8.0	48.4±4.5†	14.1±1.3†	14.0±1.0†	10.7±0.9
Control	7.0±0.3	114.7±3.6	85.7±9.2	67.8±0.0	43.4±5.7	18.0±3.3

Values are mean±SE.

* $P<0.05$, † $P<0.01$ vs control group.

TABLE 4. Animal Body Weight Loss

Groups	% of Preischemic Body Weight					
	Before ischemia	4 d	7 d	14 d	21 d	28 d
Sildenafil 2 mg/kg, 2 h	100	87.8±2.3	86.6±2.5†	89.8±4.7*	88.2±3.7†	105.2±3.0*
Sildenafil 5 mg/kg, 2 h	100	84.6±1.8	81.8±2.5*	84.7±3.0	85.2±3.5*	101.3±4.3
Sildenafil 2 mg/kg, 24 h	100	88.0±0.2	84.0±1.8†	89.2±2.5*	99.5±2.0*	107.2±2.5*
Control	100	74.7±1.1	73.8±1.1	77.6±3.4	81.5±4.7	82.4±4.6

Values are mean±SE.

* $P<0.05$, † $P<0.01$ vs control group.

PDES is an important enzyme for the hydrolysis of cGMP.² Our observations of PDES mRNA in the cortex in nonischemic rats are consistent with previous studies in which PDES mRNA and proteins were detected in rats.³ Sildenafil citrate is a potent inhibitor of PDES and causes intracellular accumulation of cGMP.² Our data show that administration of sildenafil significantly increased brain cGMP levels. In parallel with our findings, local administration of zaprinast, a relatively selective inhibitor of PDES, to rat brain slices leads to an increase of cGMP release.¹⁸ Thus, our data indicate that sildenafil affects brain PDES.

cGMP modulates vasorelaxing effects in vascular muscle.¹⁹ We found that administration of sildenafil transiently increased CBF in nonischemic rats, consistent with previous in vitro and in vivo studies. Administration of zaprinast elicits dilatation of the basilar artery in rats²⁰ and produces dilatation of dog cerebral arteries.²¹ Administration of sildenafil at a dose >5 mg/kg decreases the systolic arterial blood pressure, and the effect lasts for at least 6 hours.²² However, the effects of sildenafil on CBF likely do not provide neuroprotection because the treatment did not reduce infarct volume and the treatment was effective even when sildenafil was first administered at 24 hours after the onset of ischemia, which is far beyond the therapeutic window for neuroprotection.

Another new finding of the present study is that treatment with sildenafil significantly increases proliferation of progenitor cells in the subventricular zone and the dentate gyrus and numbers of immature neurons, as assayed by TuJ1 immunostaining. We previously demonstrated that administration of DETA/NO₂, an NO donor, significantly enhances neurogenesis.⁴ NO activates soluble guanylate cyclase and leads to formation of cGMP, while sildenafil inhibits PDES activity and results in inhibition of cGMP breakdown.^{3,23} Taken together, these data suggest that cGMP may regulate neurogenesis. Our findings are consistent with previous studies that cGMP-dependent protein kinase type I enhances sensory neuron precursor proliferation.²⁴ It is interesting to note that

neuronal progenitor cells in the subventricular zone migrate to the olfactory bulb, and after reaching the olfactory bulb, they differentiate into mature neurons.²⁵ These data are consistent with the observation that formation of olfactory memory is mediated by cGMP concentration.²⁶

cGMP levels in neurons are also involved in the modulation of dendritic and axonal guidance. Increased intracellular cGMP via semaphorin 3A can convert dendritic and axonal guidance from repulsion to attraction.^{27,28} In addition, cGMP enhances neurite outgrowth in hippocampal neurons in culture and in PC12 cells.^{29,30} Furthermore, aged rats exhibit a decrease in the basal levels of cGMP as a consequence of a more active degradation of cGMP by a phosphodiesterase in the aged brain compared with the adult brain.³¹ Decreases in NO and cGMP synthesis in aged brain may have important functional implications in the

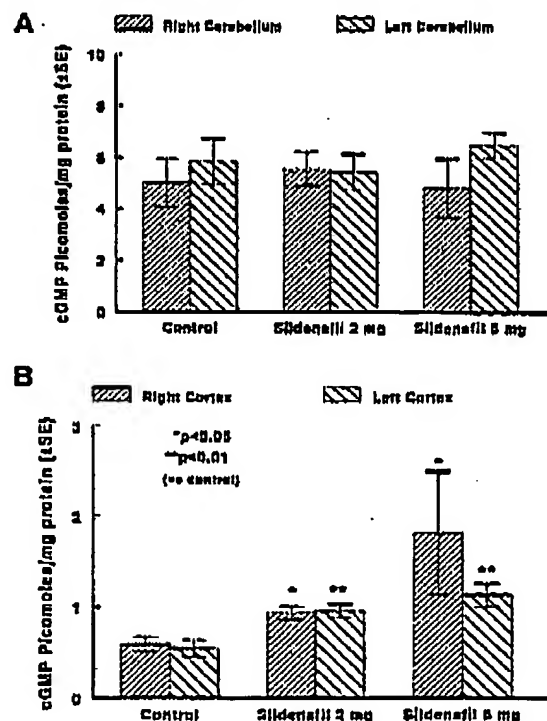


Figure 2. Levels of cGMP in the cerebellum (A) and cortex (B) after treatment with sildenafil in nonischemic rats ($n=6$; $n=10$ in control group).

TABLE 5. Infarct Volume

Groups	Infarct Volume, %
Sildenafil 2 mg/kg, 2 h	35.2±3.3
Sildenafil 5 mg/kg, 2 h	37.7±4.3
Sildenafil 2 mg/kg, 24 h	35.5±0.0
Control	38.3±1.7

Infarct volume is presented as mean±SE percentage of lesion relative to the contralateral hemisphere.

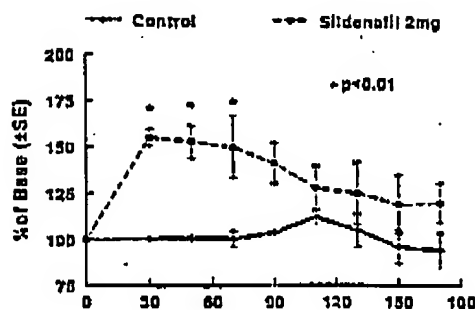


Figure 3. Localized CBF in rats treated with sildenafil (n=6) or control (n=3).

processes of learning and memory.³¹ Neurogenesis may translate into functional improvement.^{32,33} For example, mice with a high rate of neurogenesis in the dentate gyrus exhibit enhanced performance on a hippocampal-dependent task, whereas a decreasing rate of neurogenesis is correlated with impairment on such a task.^{34–36} Therefore, enhancement of neurogenesis may contribute to functional recovery after treatment with sildenafil.

In summary, the results of this study demonstrate that administration of sildenafil after stroke enhances functional recovery and augments neurogenesis in the rat.

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